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Factors determining calcium activation or inhibition of soybean lipoxygenase

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INTRODUCTION

Soybeans have great present use and future potential as a high-protein food source for humans. Soybeans are also the richest known source of the enzyme lipoxygenase (E.C. 1.13.1.13), which catalyzes the oxygenation of polyunsaturated fatty acids. The enzyme is therefore at least partially responsible for the formation of undesirable flavors in foods made from soybeans. Lipoxygenase catalyzes the co-oxidation of carotenoids such as β-carotene and has been used in breadmaking to bleach wheat flour (Balls et al., 1943). Due to the shortage of protein for human food in the world, the large production of soybeans in the U.S. and elsewhere as well as the already existing markets, it seems likely that soybeans will gain increasing favor as a source of relatively cheap, high quality protein for human food. It therefore would be useful to understand more about the soybean lipoxygenase because of its role in hindering acceptance of nutritious foods due to flavor problems.

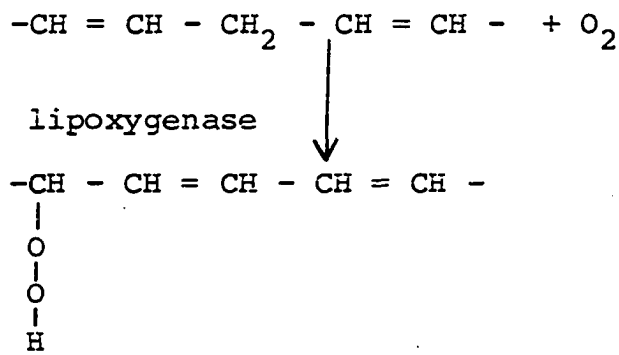
Restrepo (1971) showed that calcium activated a crude extract of soybean lipoxygenase but not a commercial preparation. Also, he reported that the enzyme was activated maximally when calcium was present in the reaction equimolar with linoleic acid. Yamamoto et al. (1970) reported two isoenzymes, one of which was activated by calcium; the other

being inhibited. There was a strong possibility that calcium had some effect on substrate, since the calcium concentration giving maximum activation varied with substrate concentration used but not with enzyme concentration. Also, since some enzyme preparations were activated by calcium and some were not, it appeared that the type of enzyme used was critical in showing calcium activation. My study was undertaken to obtain further information on the mechanism of the calcium effect by studying the effect that calcium has on the initial rates of reaction catalyzed by various enzyme preparations.

LITERATURE REVIEW

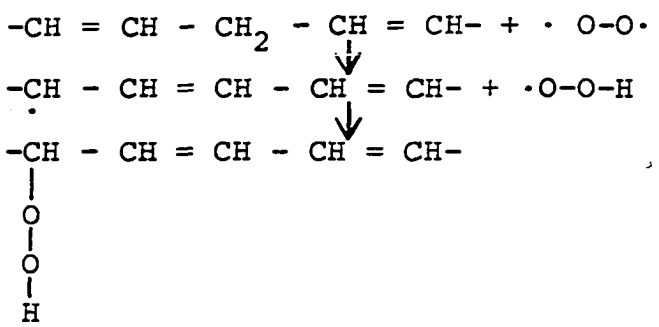
The Lipoxygenase-catalyzed Reaction and its Mechanism

Lipoxygenase uses as its primary substrates an unsaturated fatty acid containing a methylene-interrupted pentadiene structure, and molecular oxygen:

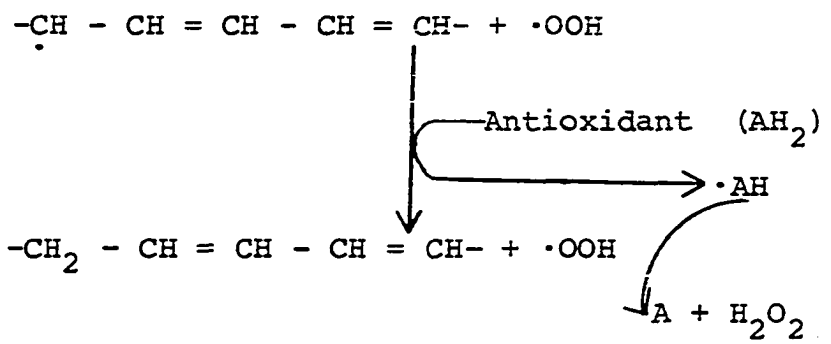


The product of the reaction is a conjugated hydroperoxide derivative of the fatty acid. The action of lipoxygenase is inhibited by commonly-used antioxidants such as nordihydroguaiaretic acid, propyl gallate and α-tocopherol (Tappel, 1963). This fact was used by some workers as presumptive evidence for a free-radical chain mechanism of lipid oxidation where the enzyme merely initiated the reaction by forming a free radical from substrate and did not enter into the reaction further. Several other plausible mechanisms for the reaction have been advanced.

Tappel et al. (1952) postulate a simple reaction mechanism involving formation of a biradical from linoleate and oxygen:



They offer several arguments against a chain reaction mechanism. First, the initial rate of linoleate oxidation is proportional to the concentration of enzyme and not to the square root of enzyme concentration as it is for a chain reaction. Second, the product formed by lipoxygenase action has a larger extinction coefficient than the autoxidized product. This is probably due to a specific geometric configuration of the double bond system in the enzyme product. Third, the action of antioxidants is readily explained by mechanisms not involving chain reactions, since nordihydroguaiaretic acid can be oxidized by the enzyme even while preventing the oxidation of linoleate:



Fourth, a maximum velocity is approached with increasing substrate concentration, in harmony with other enzyme reactions. The formation of an enzyme-substrate complex is a more satisfactory explanation of this phenomenon than is a chain mechanism, because of its simplicity. Fifth, the lipoxygenase reaction does not show an induction period. (However, other investigations, including the present one, have shown an induction period under certain conditions). Privett et al. (1955) ruled out a nonenzymatic chain mechanism because they found the reaction product to be optically active.

Tappel et al. (1953), studied further the effect of antioxidants and also temperature upon the lipoxygenase-catalyzed oxidation of sodium linoleate. They found that the phenolic antioxidants were inhibitory though to varying degrees. These workers determined the rate of reaction in the absence of inhibitors at several temperatures and from these data calculated an activation energy by use of the empirical Arrhenius equation. The activation energy was found to be 4.3 kcal/mole which was compared to the previously reported values of 7.6, 11.0 and 15.4 for pancreatic lipase, yeast invertase and trypsin, respectively, enzymes they considered to be important in food deterioration. However, the reaction rates in the solid state below 0°C were less than 1% of those in the liquid state at the same temperature. Thus freezing appeared to be a good means of protection against lipoxy-

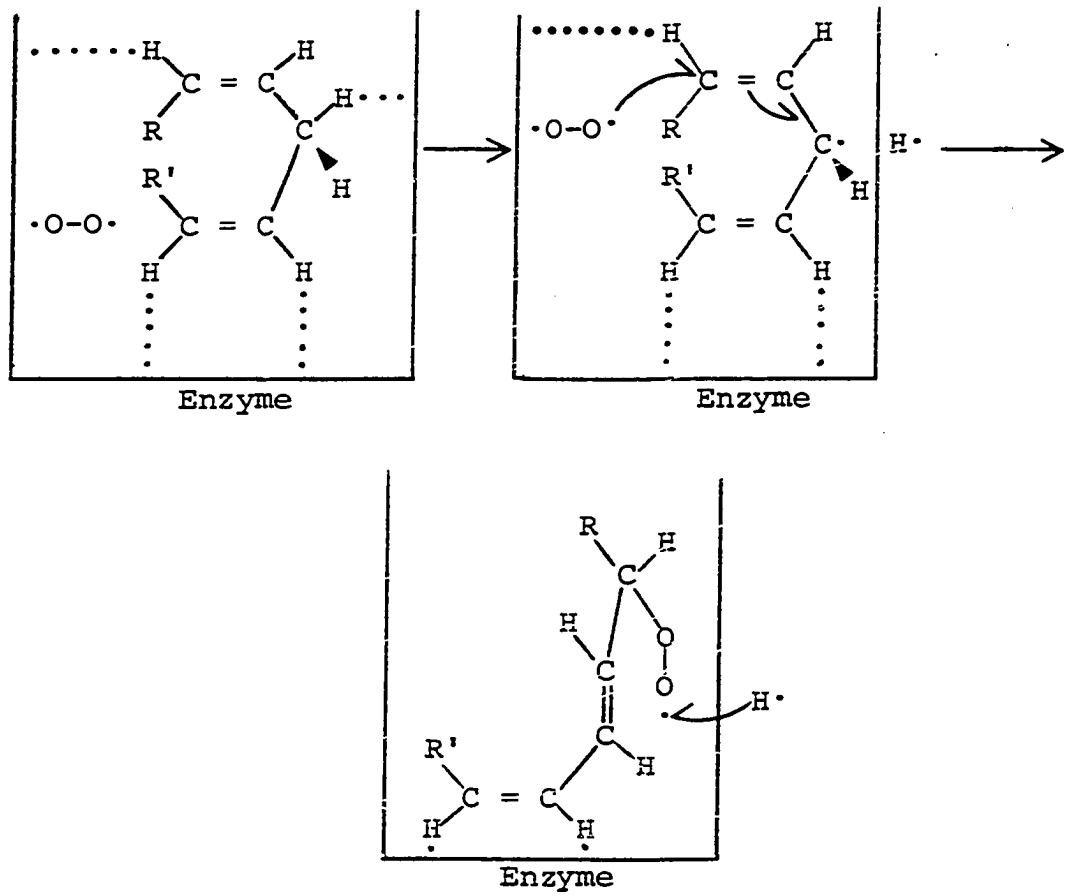
genase-catalyzed oxidations.

Fridovich and Handler (1960) found that free radicals are produced during the action of lipoxygenase. Their test for free radicals involved the aerobic oxidation of sulfite which is a chain reaction initiated by free radicals and therefore provides a sensitive test.

Walker (1963) obtained evidence for the existence of a thionyl radical on the soybean enzyme during the course of the reaction. The free radicals were determined by electron paramagnetic resonance.

Siddiqi and Tappel (1957), based on responses to p-mercuribenzoate and glutathione, found evidence for the existence of essential thiol groups in urd bean and mung bean lipoxygenases but not in soybean, wheat, pea or peanut lipoxygenases. This indicated the possibility of more than one type of lipoxygenase in nature. This point will be discussed in detail later. These workers suggest that the apparent low values for the extinction coefficient at 233 nm of the hydroperoxide they produced may be due to its breakdown, especially in crude extracts that could contain, for example, hematin compounds. Holman and Bergstrom (1951) found no evidence for prosthetic groups in soybean lipoxygenase. No effect of ferricyanide, iodoacetate or maleate

was found by Siddiqi and Tappel (1957), so they postulated a mechanism in which the enzyme itself, as opposed to a metal or coenzyme, acts as an electron sink. The mechanism includes oxygen in the normal, biradical state:



Nordihydroguaiaretic acid could act by substituting for linoleate as a free radical source for the enzyme. Co-oxidation of polyphenols and carotene could be caused by hydrogen abstraction by the peroxy free radical.

Surrey (1964) used Tween 20 as a solubilizer for linoleic

acid and found pH optima of 7.0 and 6.0 for a commercial soybean enzyme and for a crude extract from soybean meal, respectively. This is an indication of more than one isoenzyme in soybeans. Surrey used a new assay method which included solubilization of the linoleic acid substrate with NaOH. He found that increasing amounts of Tween 20 caused inhibition of enzymatic activity, expecially at the higher pH values. Also, he was among those who have studied lipoxygenase activity in germinating beans and found three distinct periods of decline in enzymatic activity during the first 80 hours of germination.

Wilkens and Lin (1970) isolated 80 volatile compounds from soy milk. Forty of these compounds were identified, the majority being aldehydes, ketones and alcohols. Mattick and Hand (1969) state that ethyl vinyl ketone, in particular, has a "beany" flavor. They propose a mechanism for the formation of ethyl vinyl ketone from linolenic acid hydroperoxide. The studies of Mattick and Hand (1969) indicate the flavor can be improved by inactivation of lipoxygenase prior to its reaction with the lipids present in the bean. This inactivation was achieved by grinding the beans in 80° water.

Vioque and Holman (1962) isolated isomeric ketodienes as secondary oxidation products of lipoxygenase action upon

linoleic acid. These ketodienes corresponded to the 9- and 13-hydroperoxides formed from lipoxygenase action on ethyl linoleate. The 13-hydroperoxide appeared to be prevalent with the use of a crude soybean extract.

Hamberg and Samuelsson (1965) found that an ω-6 double bond in the substrate was necessary for activity of their commercial soybean lipoxygenase. However, both the 9- and 13-hydroperoxide were formed from linolenic and linoleic acid in a 3:7 ratio. The ω-6 hydroperoxide product was also predominant using arachidonic acid, eicosa - 5, 8, 11, 14, 17 - pentaenoic acid and docosa - 10, 13, 16 - trienoic acids as substrates.

Hamberg and Samuelsson (1967) used a stereospecifically tritium-labeled substrate, (13 L-^{3}H, 3-^{14}C) 8, 11, 14 eicosatrienoic acid, to study the positional and stereochemical specificity of the reaction catalyzed by crystalline lipoxygenase (Fluka). They found that the 15L-hydroperoxy 13-trans isomer was formed overwhelmingly and that the removal of the ω-8 hydrogen was stereospecific. Furthermore, the large isotope effect, determined by measuring the ratio of ^{3}H to ^{14}C in unreacted substrate, suggested that the hydrogen abstraction is the initial step in the reaction.

Egmond et al. (1972) used a commercial soybean lipoxygenase and a partially-purified corn germ lipoxygenase to study the stereochemistry of the lipoxygenase reaction. They

synthesized stearate labeled with tritium at the L position of carbon 11. This was incubated along with [1-^{14}C] stearic acid in a culture of the alga Chlorella vulgaris to produce a dual labeled methyl linoleate. The ratio of ^{3}H:^{14}C was used to evaluate the amount of ^{3}H label retained in the product. Eighty percent of the product of the soybean enzyme was 13-L-hydroperoxide. With the 13-L-hydroperoxide product, in spite of the isotope effect noted also by Hamberg and Samuelsson (1967), 85 percent of the ω-8 hydrogen removed was the tritium label. Twenty percent of the product from the soybean enzyme was 9-hydroperoxide. In this product only 41 percent of the ω-8 hydrogen removed was the tritium label, and the abstraction thus appeared to be nearly random and therefore nonenzymatic.

Gardner and Weisleder (1972) offered proof for the trans-11 double bond in the hydroperoxide formed by soybean lipoxygenase. They used nuclear magnetic resonance spectroscopy and found that the 13-hydroperoxy-trans-11 isomer was formed predominantly from both linoleic and linolenic acid with a commercial lipoxygenase preparation (Pierce Chemical Company, Rockford, Illinois). They also got 17 percent 9-hydroperoxide from linoleic acid. Of the three isomers other than the 13-hydroperoxide possible from linolenic acid, mass spectral data indicated a slight specificity toward the 9-hydroperoxide. These facts they took to

be some indication of the presence of an isoenzyme.

Assay Methods

There are three ways of assaying for the activity of lipoxygenase (Holman, 1955). The first method is measurement of oxygen consumption manometrically or polarographically. The second method is measurement of the co-oxidation of an oxidizable pigment such as β-carotene. The third method is a spectrophotometric determination of the increase in absorbancy at 233 nm caused by the accumulation of conjugated diene. Holman (1955) states that the manometric methods are not to be recommended because the rate of oxygen uptake is sometimes not proportional to the amount of enzyme extract used, possibly because of something he calls the "activation" effect of emulsified substrate. He recommends that petroleum ether rather than other fat solvents be used to extract fat from soybeans, in order to avoid enzyme inactivation (Holman and Bergstrom, 1951). Holman (1955) also reports that the highest specific activity of the material extracted from soybeans is obtained when the extraction is done at pH 4.5.

Mitsuda et al. (1967a) reported crystallization of the lipoxygenase enzyme from soybeans and the use of a polarographic assay which they claimed was superior to the other assay methods. The rod-shaped crystals were found to be homogeneous by sedimentation velocity and electrophoresis on

cellulose acetate. The molecular weight obtained by thin-layer gel filtration on Sephadex G-100 was 102,000 ± 3,000. Their polarographic assay used a Clark type oxygen electrode. They found initial rates to be proportional to enzyme concentration up to 6×10^{-7} moles O_2 per minute in a 3 ml. reaction volume. The concentration of linoleic acid was 6.8×10^{-3} M in .1 M NH_4Cl buffer, pH 9.0. They assumed that the saturated oxygen concentration at 25°C was 2.4×10^{-4} molar. A protein amount of .58 µg gave a rate of about 120 nanomoles O_2/min. This is a specific activity of 207 µmoles min^{-1}/mg. prot. Mitsuda et al. (1967a) observed no induction period for any of the enzyme concentrations used. They imply that this is an advantage of the polarographic method and also list some other advantages:

1. Proportionality of initial rate to enzyme amount used.
2. Continual and direct measurement of one of the reactants.
3. Impure enzyme can be used.
4. Doesn't require optical clarity of the reaction mixture.

Tappel (1962) gives some standard assay conditions for use in manometric and spectrophotometric methods of analysis. For preparations low in lipoxygenase activity, he cautions that other lipid peroxidation catalysts may interfere with the

assay. This is especially true for heme proteins like cytochromes. The presence of lipoxygenase can be verified by running heat-inactivated controls and by a lipoxygenase specificity test. This test relies essentially on the fact that lipoxygenase will oxidize lincleic, linolenic and arachidonic acids but not oleic acid (Holman and Bergstrom, 1951). In fact, Tappel attributes the claim by some investigators to have found lipoxygenase in animal tissues to oxidation of lipoxygenase substrates by hemoglobin, myoglobin or cytochromes.

Enzyme Activation and Inhibition

Theorell et al. (1947b) crystallized soybean lipoxygenase and used the spectrophotometric method they had previously developed to assay the activity. This preparation was extracted from defatted soy flour at pH 4.5 and heated to 63°C for five minutes. After ammonium sulfate and alcohol fractionation, the most active fraction was separated by electrophoresis and then crystallization was achieved by slow dialysis against increasing ammonium sulfate concentration. The crystals were homogeneous by sedimentation and diffusion measurements and the molecular weight was estimated at between 90,000 and 100,000. The authors state that an iron determination was done on a nearly pure sample and that "any iron present

must be present as an impurity." Fluoride, cyanide, azide and pyrophosphate did not inhibit the enzyme.

Pistorius and Axelrod (1973) have found evidence for the presence of iron in soybean lipoxygenase and its essentiality for catalytic action.

Balls et al. (1943) used the rate of bleaching of a carotene suspension to follow the purification of the enzyme. Sumner and Dounce (1939) had previously shown a co-oxidative activity of soybean water extracts acting upon carotene in the presence of unsaturated fatty acids or their triglycerides. Balls et al. (1943) found that an activator, which behaved much like a peptide but was not isolated, increased the carotene oxidase activity when increased in the reaction along with primary substrate. That is, the substrate inhibition of the enzyme was reversed by the activator. They took this fact to be evidence for the "accelerating substance" acting upon substrate. Substrate inhibition was the term used for a reduction in activity of the enzyme with increased substrate concentration. Balls et al. (1942) had shown potent inhibition of the soybean enzyme by purothionin, and reversal of inhibition by an increase in substrate concentration. Purothionin was thought to inhibit because of surface effects in the fat suspension and it was postulated that the activator also acts by surface effects.

Interestingly, the isolation procedure of Balls et al.

(1943) included a dialysis against tap water to remove ammonium sulfate. This might have added calcium to the enzyme, and as will be seen, calcium can activate lipoxygenase. Activating substance was present in many natural sources. The concentrated activator contained 16.2 percent N and .068 percent P and was inactivated by papain. It absorbed at 270 to 285 nm but not at 260 nm. In order for the activator to be effective, it had to be added to the enzyme or to the fat suspension before adding enzyme. The enzyme itself contained no activator or a very small amount. Addition of activator resulted in a first order reaction in enzyme. Without activator, mixed order and zero order kinetics were observed. As with calcium activator, varying the amount of enzyme did not change the amount of activator required for optimum activity but varying the substrate concentration did. Purification of the activator resulted in a slower initial rate of reaction but a higher total peroxide production. This indicates removal of some other factor during activator purification. If purothionin was added to the substrate, it was very inhibitory, but not if added to the enzyme in the presence of activator. Also, the inhibitory action of purothionin was dependent on the amount of substrate present. Phosphate buffer was necessary for the inhibition by purothionin. Order of addition of buffer salt was important, no action of purothionin being observed

if salt was added after the enzyme.

Tappel et al. (1952) did further work to elucidate the mechanism of soybean lipoxygenase. Using sodium linoleate as substrate under controlled temperature and oxygen concentration, the reaction velocity was linear with respect to enzyme concentration and the enzyme did not show an induction period. With methyl linoleate as substrate, the addition of Tween was necessary to get a linear relationship between velocity and enzyme concentration. Presumably the Tween solubilizes substrate so that the enzyme remains saturated when present at the higher levels.

O'Reilly et al. (1969) noted that cauliflower lipoxygenase does not conform well with Michaelis-Menten kinetics. The Km at low substrate concentration was found to be 5×10^{-5} M, but at substrate concentrations above 1×10^{-4} M, an unexpectedly high initial rate was seen. Manometric measurements (necessary because of presumed substrate insolubility at high concentration) gave a Km of about 2×10^{-3} M at substrate concentrations above 1×10^{-2} M. The enzyme is progressively more activated at substate concentrations above 1×10^{-4} M. O'Reilly et al. suggest two possible explanations for this behavior. First is an allosteric activation. The second possibility is related to the formation of substrate micelles above 1×10^{-4} M. According to this theory, enzyme activation occurs by absorption to

micelles. The activation may be due to a suitable ordering of substrate molecules or to a change in enzyme structure upon binding to the micelle.

Allen (1968) claimed that micelle formation had little effect on the Km of the soybean lipoxygenase. The critical micelle concentration (CMC) was about 2×10^{-5} M as measured by surface tension. This was also the approximate Sm (extrapolated value of the two limbs of the curve of velocity plotted against substrate concentration). The addition of methanol or dioxane at different pH's varied both the CMC and the Sm. Michaelis-Menten kinetics were observed between 10 and 150 μM linoleate in all cases. Above these concentrations, however, Michaelis-Menten kinetics did not appear to apply. But since the Km does not change over the range of the CMC, it seems that the enzyme is not hindered or helped by the formation of micelles.

Kies (1947) isolated a crystalline polypeptide from soybeans which activated lipoxygenase by as much as 300 percent. A similar substance was found in gum arabic, a gum used by Cosby and Sumner (1945) to stabilize their substrate emulsions. Kies (1947) suggests that the activator acts on the substrate rather than enzyme, since the enzyme is activated with linoleic acid as substrate but not with methyl linoleate as substrate.

Balls et al. (1946) state that the failure of Cosby

and Sumner to show activation is due to their use of gum arabic. Balls et al. (1946) also found that the quantity of activator necessary to give maximum activation is not dependent on the amount of enzyme but on the amount of substrate.

Smith and Sumner (1948) prepared the activator of Balls et al. (1943) but found no activation. These results were true even in the absence of gum arabic but in the presence of bentonite or sodium dodecyl sulfate.

Walsh et al. (1970) obtained kinetic data indicating that L-ascorbate is a competitive inhibitor in lipoxygenase from varieties of durum wheat. This has practical application in controlling the co-oxidation of β-carotene and thus the color of pasta.

Blain and Shearer (1965) obtained data for the competitive inhibition of soybean lipoxygenase by certain long-chain polyacetylenic fatty acids. Eicosa - 5, 8, 11, 14-tetraynoic acid caused more than 50% inhibition of the enzyme when present at .05 times the linoleate concentration and was thus the most potent competitive inhibitor yet reported. The corresponding alcohol gave only half as much inhibition as the tetraynoic acid, suggesting that the carboxyl group confers greater affinity for the enzyme than does the hydroxyl. Nordihydroguaiaretic acid, in increasing amount, increased the length of the induction period and lowered the extent of reaction. It was not considered to be a competitive in-

hibitor, a reasonable conclusion in view of the dissimilarity of its structure to that of the substrates.

Haining and Axelrod (1958) verified that an induction period occurs during the lipoxygenase-catalyzed oxidation of sodium linoleate. Although an induction period might be construed as representing a chain reaction mechanism, the work of Privett et al. (1955) indicating optical activity in the product, seemed to rule out a nonenzymatic chain reaction. Haining and Axelrod (1958) found that the induction period (lag period) can be abolished by the addition of small amounts of linoleate or linolenate hydroperoxides to the reaction mixture.

Smith and Lands (1972) found that lipoxygenase is destroyed during its oxygenation of fatty acid substrate. The destruction of the enzyme is first order with respect to enzyme and the rate constant has a characteristic value for each fatty acid substrate. The rate of inactivation was much greater when the enzyme was pre-incubated with product hydroperoxide and fatty acid than when incubated with fatty acid alone. The authors postulate a model in which the enzyme molecule, present in a complex along with product hydroperoxide, fatty acid, and oxygen can either produce another molecule of product or become inactive.

Smith and Lands (1972) also confirmed that there exists, in the oxygenation of fatty acids, a lag period which can

be eliminated by adding product hydroperoxides.

Downing et al. (1970) found that acetylenic analogs of substrate fatty acids are irreversible inhibitors. Preincubation of the enzyme with 10^{-6} M 5, 8, 11, 14-eicosatetraynoic acid showed a slow but irreversible attack on the enzyme. The enzyme was completely inactivated after 40 minutes. If the acetylenic analog was put in the reaction mixture, the reaction ceased after about 40 percent conversion of substrate.

Mitsuda et al. (1967b) found that 10, 12 linoleic acid, a competitive inhibitor, protects the enzyme against H_2O_2. On the other hand alcohols, though they inhibited the enzyme were ineffective in protecting the enzyme against inactivation by H_2O_2. The alcohols were reversible, mixed inhibitors of the reaction. Plots of the binding constant for inhibitory alcohols versus the temperature showed a positive entropy for binding, implying a hydrophobic interaction of alcohol and enzyme (Mitsuda et al., 1967b). Also, binding increased with increasing chain length, implying hydrophobic binding of alcohol to enzyme. The mixed inhibition was taken to mean that alcohol binds close to the active site of the enzyme but not at the active site. The hydrophobic binding site for the alcohol was presumably the one used by substrates. Since alcohol did not affect the H_2O_2 inactivation, Mitsuda et al. (1967b)

concluded that H_2O_2 probably acts at the active site.

Isoenzymes and Subunits

Koch et al. (1958) showed evidence for the existence of two isoenzymes when they separated two fractions containing lipoxygenase activity from defatted soy flour. Fraction H showed greater activity on trilinolein than on linoleic acid. Fraction J was more active on linoleic acid than on trilinolein at pH 8.1, though the reverse was true at pH 5.3. Fraction J was more specific in its substrate requirements than fraction H at pH 8.1. However, at pH 5.3, fraction J was actually less active than fraction H on linoleic acid. Fraction H was active on methyl linoleate while fraction J had very little activity. Koch et al. (1958) found differences in the two fractions with respect to solubility and optimum pH as well as substrate specificity and reaction rates. Their isolation procedure for both fractions included the use of $CaCl_2$ (3.4×10^{-2} M) to precipitate inactive protein from the crude extract.

Guss et al. (1967) subjected crude aqueous extracts of wheat and soybean to polyacrylamide gel electrophoresis. They then used a specific staining procedure to identify bands of lipoxygenase on the gels. The soybean extract contained four isoenzymes that were separated on the gels. The staining procedure involved first, the incubation of the gels

with linoleate. Subsequently the gels were treated with acidic potassium iodide. The iodine formed by the linoleate hydroperoxide reacted with the .5 percent starch present in the gels to give brown to blue bands identifying the zones of lipoxygenase activity on the gels.

Kies et al. (1969) claim that the enzyme responsible for carotene bleaching is distinct from the classical Theorell enzyme. They show data indicating that the Theorell enzyme is not as active as crude soybean extract (CSE) in bleaching carotene and is more resistant to heat. Also, the CSE loses no activity on linoleic acid upon heating while its activity on carotene goes almost to zero. On the other hand, the activity of the Theorell enzyme on linoleic acid changes little with heating and on carotene not at all.

Yamamoto et al. (1970) isolated two isoenzymes of soybean lipoxygenase after learning that only about one-third of the activity of a crude soybean extract could be crystallized as the classical or Theorell enzyme. Extraction in water at neutral pH rather than at pH 4.5 was selective for the new isoenzyme. The two isoenzymes were separated on an ion exchange column and were differentiated by polyacrylamide gel electrophoresis, pH activity profile and response to calcium. Further details on the catalytic behavior of the two isoenzymes will be found in the next section of this literature review.

Christopher et al. (1970) isolated a distinct lipoxygenase, different from the well-known lipoxygenase of Theorell. The distinction was made on the basis of separation on a DEAE Sephadex column, disc gel electrophoresis, pH activity profile, substrate specificity and heat stability. The new isoenzyme, which they called lipoxygenase 2, had no activity at pH 9.0 and this characteristic was used to separate it from the Theorell enzyme (lipoxygenase 1) which was isolable in greater quantity. The anion exchange and electrophoretic mobilities indicated that lipoxygenase 2 is less negatively charged than lipoxygenase 1. Its half-life at 69°C was about .7 min. as compared with 25 min. for lipoxygenase 1.

Christopher et al. (1972) isolated a third isoenzyme, lipoxygenase 3, from soybean by DEAE Sephadex and isoelectric focusing. They also found that lipoxygenase 2 was stimulated by calcium but that lipoxygenase 3 was increasingly inhibited by .3 to 3 x 10^{-3} M calcium at pH 6.8 with linoleic acid present at 1.23 x 10^{-3} M.

Stevens et al. (1970) purified a soybean lipoxygenase to essential homogeneity as judged by acrylamide gel electrophoresis and ultracentrifugation. Treatment of this protein with sodium dodecyl sulfate resulted in dissociation into two subunits of equal molecular weight as determined by sedimentation-velocity ultracentrifugation. However, dis-

sociation was incomplete, as it was also with 6M guanidine hydrochloride and .5 percent mercaptoethanol. Stevens et al. (1970) found four sulfhydryl groups and two disulfide bonds in the molecule but they agreed with Tappel (1963) that thiol reagents do not inhibit the lipoxygenase.

The Effect of Calcium on the Lipoxygenase-catalyzed Reaction

Calcium activation of the enzyme may have first been noticed by Sullman (1941), cited in Balls et al. (1943), when he observed that dialysis caused partial inactivation of the lipoxygenase, possibly due to removal of an activating substance. Balls et al. (1943) isolated a protamine from soybeans which they claimed was the activator. The optimal activator concentration varied with substrate concentration, just as occurs with calcium (Restrepo et al., 1973). Cosby and Sumner (1945), however, were not able to show an activating substance in soybeans. Balls et al. (1946) reported concentrating an activator which they thought was a mixture of peptides. Theorell et al. (1947a) found activator unnecessary but Kies (1947) isolated a polypeptide activator. Franke and Frehse (1953) did not find activation with soybean extracts but when barley extracts were dialyzed against tap water, a definite increase in lipoxygenase activity resulted. Oxygen uptake rate was also increased upon addition of calcium

to the enzyme in an amount equal to that added by the dialysis. This amount was approximately 4.5×10^{-3} M. This enzyme solution was then diluted 1:10 in the reaction mixture, making a final calcium concentration of 4.5×10^{-4} M. No activation was found upon dialysis against distilled water, indicating that the activation was not due to removal of an inhibitor.

Koch (1968) found that navy bean lipoxygenase was activated by calcium with linoleic acid as substrate at pH 7.5. In his hands, this pH was normally associated with a low enzyme activity. He found and notes briefly in his paper that soybean extracts also possess a calcium ion stimulated lipoxygenase. Sodium deoxycholate further increased the activity in the presence of calcium but EDTA reversed the activation. The beans were extracted by Koch (1968) according to the procedure of Dillard *et al.* (1960) which uses distilled water extraction with the addition of 64 mg. of $CaCl_2$ per ml. of extract to precipitate inactive protein.

Holman *et al.* (1969) showed a broadening of the substrate specificity pattern with calcium present at a 1×10^{-7} M concentration. That is, compared to linoleic acid as a standard in the presence or absence of calcium, other methylene-interrupted cis, cis-octadecadienoic acids gave a higher percentage activity when calcium was present than when calcium was absent. This was true when the first double bond occurred between carbon 5 and carbon 11. Below carbon 5,

no activity occurred with or without calcium. Above carbon 11, calcium actually narrowed the specificity. Holman et al. (1969) studied substrate specificity further, in the presence of calcium. The highest activity was obtained using linoleic acid. Other substrates with high activity contained ω-6 and ω-9 double bonds. However, some substrates with low activity had the same double bond arrangement. There was no apparent effect of activity based on the distance of the double bonds from the carboxyl end of the fatty acid molecule. However, α-methyl arachidonic acid gave no activity, in contrast to a high rate of activity with the parent acid. This Holman et al. took to mean that a sterically unhindered carboxyl group was required in the substrate and suggested that calcium acts by mediating binding of substrate to enzyme.

Christopher et al. (1970) extracted soybean flour in the presence of 10^{-4} M calcium and separated two isoenzymes on a DEAE-Sephadex column. Lipoxygenase 2 was inactive at pH 9.0 but active at pH 6.8 in the presence of 5.9×10^{-4} M calcium. The opposite was true of lipoxygenase 1.

Yamamoto et al. (1970) reported the isolation of an isoenzyme (lipoxygenase b) of soybean lipoxygenase which was activated by calcium ion at the higher substrate concentrations used. This activation was apparently a reversal of substrate inhibition. The effect of calcium at the lower substrate con-

centrations was variable. In addition, Yamamoto et al. (1970) got results indicating that the Theorell enzyme (lipoxygenase a) is competitively inhibited by calcium. These findings are important in that they may help to reconcile some of the conflicting information about activation of the lipoxygenase enzyme. Yamamoto et al. (1970) began looking for isoenzymes when they discovered that only about one-third of the total lipoxygenase activity in a crude extract could be recovered in crystalline form as the classical enzyme. Extraction in water rather than at pH 4.5 yielded an enzyme component with an optimum activity at pH 7.0 as opposed to 9.0 with the enzyme extracted at pH 4.5. The water extract also contained more of the lipoxygenase b as shown by gel electrophoresis and was stimulated by calcium between pH 5.5 and 9.5. However, the activities reported for lipoxygenase b were 2 orders of magnitude less than those reported for lipoxygenase a.

Koch et al. (1971), working again with the navy bean enzyme, found that calcium, magnesium and manganese are activators. Calcium was the most active of the three metals in stimulating lipoxygenase activity. Ferric ion and cupric ion were inhibitors for the calcium-stimulated activity. Koch et al. (1971) observed different pH optima in the presence and absence of calcium. They ascribed this phenomenon to the presence of two isoenzymes or to two ionizable groups.

They did not use calcium during extraction of the enzyme. However, the navy bean and soybean extracts contained calcium which they easily removed by passage of the enzyme through a Sephadex G-25 column. This treatment also inactivated the enzyme, which was easily reactivated by the addition of calcium. The optimum calcium concentration for the soybean lipoxygenase occurred over a broad range in phosphate buffer. A sharp decline in activity occurred at the calcium concentration associated with the development of turbidity in the reaction mixture. This could indicate a calcium effect upon availability of the substrate to enzyme.

The navy bean enzyme responded positively to added calcium up to 3×10^{-4} M calcium, above which concentration of calcium it was inhibited. Koch et al. (1971) offer two calcium binding sites as an explanation for this; one activating site and one inhibiting site. By varying the order of addition of calcium and enzyme to the reaction, these investigators found evidence that an enzyme-linoleate complex can form in the absence of calcium. However, this complex is not much affected by calcium added after enzyme, in contrast to the cases where calcium is added before enzyme or added directly to the enzyme before introduction to the reaction mixture.

Koch et al. (1971) observed a flocculant precipitate which settled out of the clear supernatant of a navy bean

extract and which they named cold-insoluble material (CIM). They assumed this was a protein-phytate since it was solubilized by $CaCl_2$ and because it enhanced the calcium activation of lipoxygenase activity in navy bean extracts. Although CIM also forms in soybean extracts held below 5°C, Restrepo et al. (1973) were unable to show any effect of phytates in calcium activation of soybean extracts. Though the procedure for the removal of phytates made the enzyme preparation refractory to calcium activation, the subsequent addition of phytate did not restore the calcium-activated property to the extracts. Restrepo et al. (1973) therefore concluded that another substance mediating calcium activation was removed by the phytate-removing procedure. Restrepo (1971) found that crude extracts of soybeans (CSE) are activated by calcium but that a commercially purified enzyme (Sigma) is not. This is not too surprising in view of the claim of Yamamoto et al. (1970) for 2 isoenzymes. Restrepo (1971) found no calcium activation of CSE and other impure enzyme preparations with trilinolein as substrate and this is also feasible in view of the report by Koch et al. (1958) that a separate isoenzyme exists which prefers trilinolein as substrate. The initial rates of reaction found by Restrepo et al. (1973) were proportional to enzyme concentration, especially at pH 8.0. However, the optimal calcium concentration was independent of enzyme concentration. The maximum

calcium activation was achieved when calcium was equimolar with substrate. High concentrations of calcium lowered the rate of reaction.

MATERIALS AND METHODS

Enzyme Preparations

1. Crude soybean extract:

Soybeans of the Amsoy variety, 1971 crop, were obtained from the Agronomy Department at Iowa State University. They were stored at 2°C until used. The soybeans were ground in a Wiley mill to pass a 20 mesh screen. The resulting soybean flour was defatted with several changes of hexane, dried in air and then extracted at room temperature with ten times its weight of deionized water. The slurry was stirred on a magnetic stirrer for 30 minutes at room temperature and then pressed through four thicknesses of cheesecloth to remove the larger soybean particles. The suspension was then centrifuged at room temperature for 15 minutes at 10,000 g and the supernatant was collected and refrigerated. Cold-insoluble material in the CSE was normally resuspended by shaking gently or allowing the CSE to come to room temperature before use as an enzyme source.

2. Partially purified enzyme:

Defatted soybean flour was extracted in ten times its weight of .05 M Na acetate buffer, pH 4.5. The fraction precipitating between 40 and 80% of ammonium sulfate saturation was centrifuged and resuspended in .2 M Tris buffer pH 8.0. This preparation and CSE were opalescent in appearance.

3. Purified lipoxygenases 1 and 2:

Defatted soybean flour was extracted in 10 volumes .05 M Na acetate pH 4.5 in the cold overnight. This was taken to 40% of ammonium sulfate saturation with slow addition of solid salt and stirring. After overnight storage in the refrigerator, the fraction was centrifuged at 8000 g for 15 minutes. The precipitate was resuspended in .01 M phosphate pH 6.8 and dialyzed against the same buffer overnight in the cold with 4 changes of buffer. The protein solution received a final centrifugation and was then placed on a 2.5 cm by 40 cm DEAE Sephadex A50 column and eluted in .01 M phosphate pH 6.8 with a 0-.4 N sodium chloride gradient. Fractions of 10 ml were collected and the first fraction showing activity came off the column with a peak at about fraction 66. This was lipxygenase 2. The second active peak came off at about fraction 95 and was lipoxygenase 1.

Substrates

Linoleic acid (99% pure) from Sigma Chemical Company was dissolved in 95% ethanol at the concentration of 10 mg ml^{-1}. This was used as substrate by adding it to the reaction with a microliter syringe. In experiments using variable amounts of substrate, a constant amount of ethanol was added to each reaction mixture. Methyl linoleate and trilinolein from Supelco were also dissolved in 95% ethanol for use as

substrates.

Water

Water for all uses was deionized water obtained from a commercial automatic still and then passed through a Barnstead Standard Mixed Bed Cartridge and stored in polyethylene bottles.

Chemicals

All chemicals were commercial, reagent grade products.

Assays

1. Spectrophotometric assay:

The increase in absorbancy at 234 nm (Tappel et al., 1952) was followed in a Beckman DK-II A ratio recording spectrophotometer using the time drive setting. Quartz 1 cm cuvettes were used and the enzyme and substrate were mixed by inverting the cuvettes by hand several times before placing them in the spectrophotometer. The reaction mixture in the cuvettes was not sealed off from the air. The absorbancy at 234 nm was plotted as a function of time by the spectrophotometer and the velocity of the reaction was determined from the slope using an extinction coefficient for the product of 28,000 M^{-1} cm^{-1}.

2. Oxygen uptake assay:

The presence of calcium in activating amounts in a fatty acid substrate solution results in a turbid suspension which is impossible to assay at 234 nm. Therefore, most of the

assays were done using a Yellow Springs Instrument Co. (YSI) Model 53 Oxygen Monitor connected to a Sargent-Welch SRG recorder. The YSI instrument uses a self-contained polarographic system isolated from the reaction by a gas permeable Teflon membrane. The reaction mixture is kept out of contact with air by a plunger which occupies all the space in the reaction chamber above the reaction mixture. The readout on the SRG strip-chart is in O_2 content as a percent of the original O_2 content, versus time. The O_2 content of a buffer solution equilibrated with air is taken to be the same as of pure water at the same temperature.

Washing of Reaction Vessels

The vessels were scrubbed with a commercial liquid dish detergent in distilled water and rinsed extensively with distilled water. They were then rinsed repeatedly with deionized water and dried in a drying oven. The magnetic stirrers were washed and rinsed the same way, then dried in air or with the use of Kimwipes.

Determination of Calcium in Enzyme Preparations

Calcium concentrations in the various enzyme preparations were determined with a Beckman atomic absorption accessory in conjunction with a Beckman DU Spectrophotometer. The absorbances at 4226 Å were determined and converted to calcium concentrations by referring to a standard curve.

RESULTS AND DISCUSSION

Lipoxygenase Assays

Use of crude soybean extract (CSE) as enzyme

Restrepo (1971) found that calcium increased the activity of lipoxygenase in soybean extracts but did not activate partially purified lipoxygenase (Sigma); therefore much of my work on calcium activation was done with CSE. Boiled CSE showed no oxygen uptake when incubated with linoleic acid, thus nonenzymatic catalysis of oxidation of polyunsaturated fatty acids is not detected by the assays. Also, unheated CSE showed no activity when incubated with oleic acid, so CSE contains no enzymes catalyzing the oxidation of single double bonds.

Figure 1 shows that lipoxygenase activity in CSE is proportional to the rate of O_2 uptake below a rate of 2.4 μmoles min^{-1} at 25°C in a 3 ml reaction. This exceeds the maximum rate for O_2 uptake of .223 μmoles min^{-1} recommended by the manufacturers of the YSI instrument, but since there was linearity between amount of lipoxygenase used and rate of O_2 uptake, the YSI instrument operated satisfactorily up to the rate of 2.4 μmoles min^{-1}.

Even though the upper limit on linearity of the plot of velocity versus enzyme amount exceeds the manufacturers recommendations, the limit corresponds roughly to the electrode response noted by the YSI company. They state

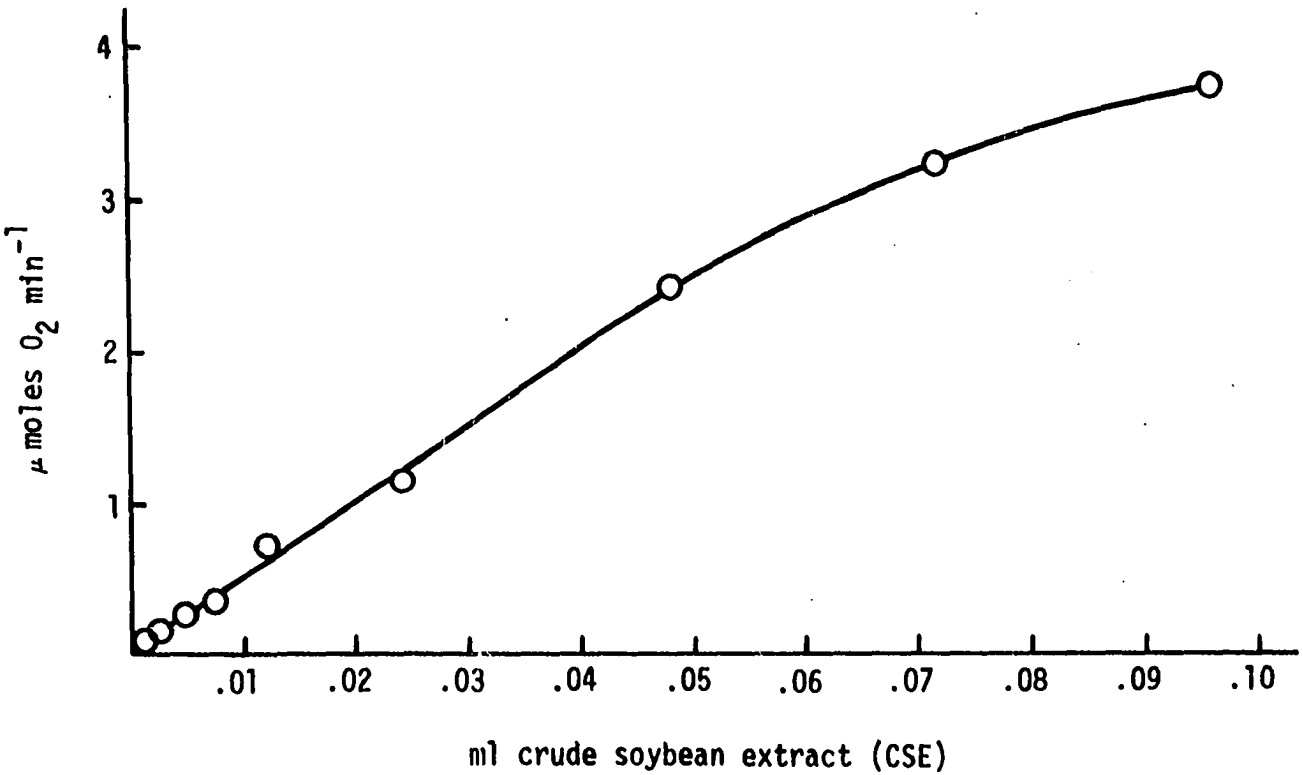


Figure 1. The dependence of observed initial velocity upon the amount of CSE used. The reactions were run in 3 ml .01 M phosphate buffer, pH 8.0 with linoleic acid present at a concentration of 1.4×10^{-3} M

that the electrode can respond to the extent of 90% of full scale in 10 seconds or 99% of full scale in 20 seconds. In my assays at 25°C, 99% of full scale in 20 seconds would be equivalent to 2.14 μmoles min^{-1}.

As other investigators have observed, lipoxygenase exhibits an initial lag when acting on linoleic acid. The lag is more prominent at pH 9 than at pH 8[1], and is more prominent when small amounts of enzyme are used. Figure 2 shows the lag with CSE and the subsequent straight line portion of the assay which was used for calculating lipoxygenase activities. The lag is not observed with lipoxygenase 2.

Substrate concentrations

Lipoxygenase acts on two substrates: molecular oxygen and polyunsaturated fatty acids. Oxygen was provided by equilibrating the reaction buffer with air and the oxygen concentration was taken to be that in pure water, 2.4×10^{-4} M at 25°C (Hodgman, 1960). Under these conditions, the reaction was zero order in oxygen as evidenced by the fact that a five-fold increase or decrease in oxygen concentration had no effect on initial rate.

Although molar concentrations can be calculated for the

[1]H. E. Snyder, Department of Biochemistry, Iowa State University, Ames, Iowa, Private Communication. 1972.

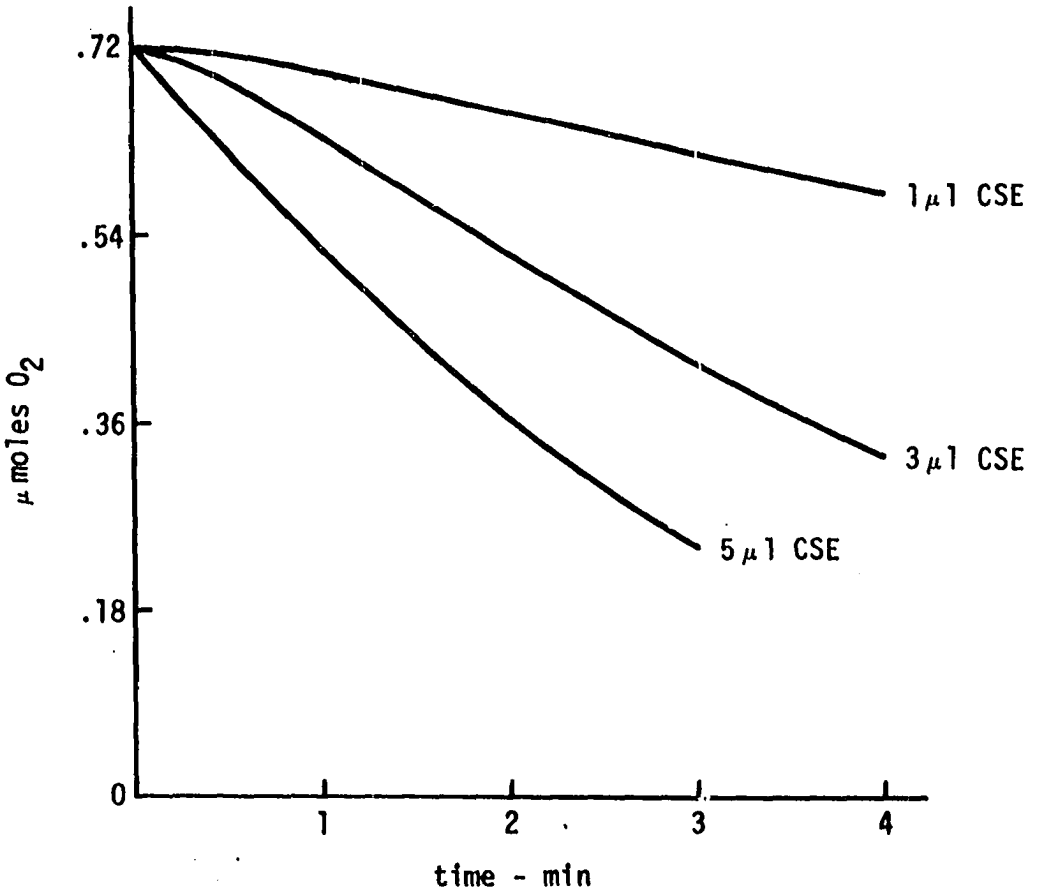


Figure 2. Plots from the SRG Recorder showing the lag period and subsequent straight-line portion. Also shown is the elimination of the lag with greater amount of enzyme. The reactions were run in .2 M borate buffer pH 9.0 at 10°C with linoleic acid present at a concentration of 3.5 x 10^{-4} M

linoleate dispersions, the actual state of the substrate is probably an equilibrium between free fatty acid or fatty acid salt and micelles of fatty acid. Allen (1968) calculated critical micelle concentration as 2×10^{-5} M based on surface tension measurements. This is 1/12 of the O_2 concentration in water and is too low to obtain an initial rate accurately under my conditions of assay.

Allen (1968) found that the enzyme is activated by substrate above the critical micelle concentration but that the Km stays constant over the range of the critical micelle concentration. It would thus seem that lipoxygenase can act equally well in the presence as in the absence of micelles. Tappel et al. (1952) also noted two Km values and substrate activation of enzyme.

As linoleate concentration increases, the surface tension decreases, and presumably the lipoxygenase activity might be affected by surface tension. However, the big decrease in surface tension occurs prior to the critical micelle concentration.

Calcium Activation

Effect of calcium on rate of reaction

Calcium is an effective activator of the lipoxygenase activity of CSE. This is illustrated in Table 1 and Figure 3. In Table 1, a calcium concentration equal to the substrate

Table 1. The effect of calcium when added to CSE immediately before initiating the reaction. The concentration of linoleic acid was 1.4×10^{-3} M in .01 M phosphate pH 8.0

Calcium concentration	μmoles O_2 min^{-1} ml^{-1}	Standard deviation (n=8)
0	30.9	3.7
1.4×10^{-3} M	61.6	3.2

concentration approximately doubles the rate of reaction. However, there is a fair amount of variability in the values for initial velocity. In Figure 3, the optimum calcium concentration is nearly twice the substrate concentration. The calcium activation declines after reaching a maximum. Excess calcium does not remove substrate from the reaction, since another experiment showed that the reaction is completed even at the high calcium levels. However, it is possible that calcium alters the availability of substrate to the enzyme.

Order of addition of reaction components

The usual order of addition of components to the reaction buffer was as follows: Calcium was added to the buffer first. Then substrate was added, and lastly the reaction was initiated by the addition of enzyme. As reported by Restrepo et al. (1973), Koch et al. (1971), Yamamoto et al.

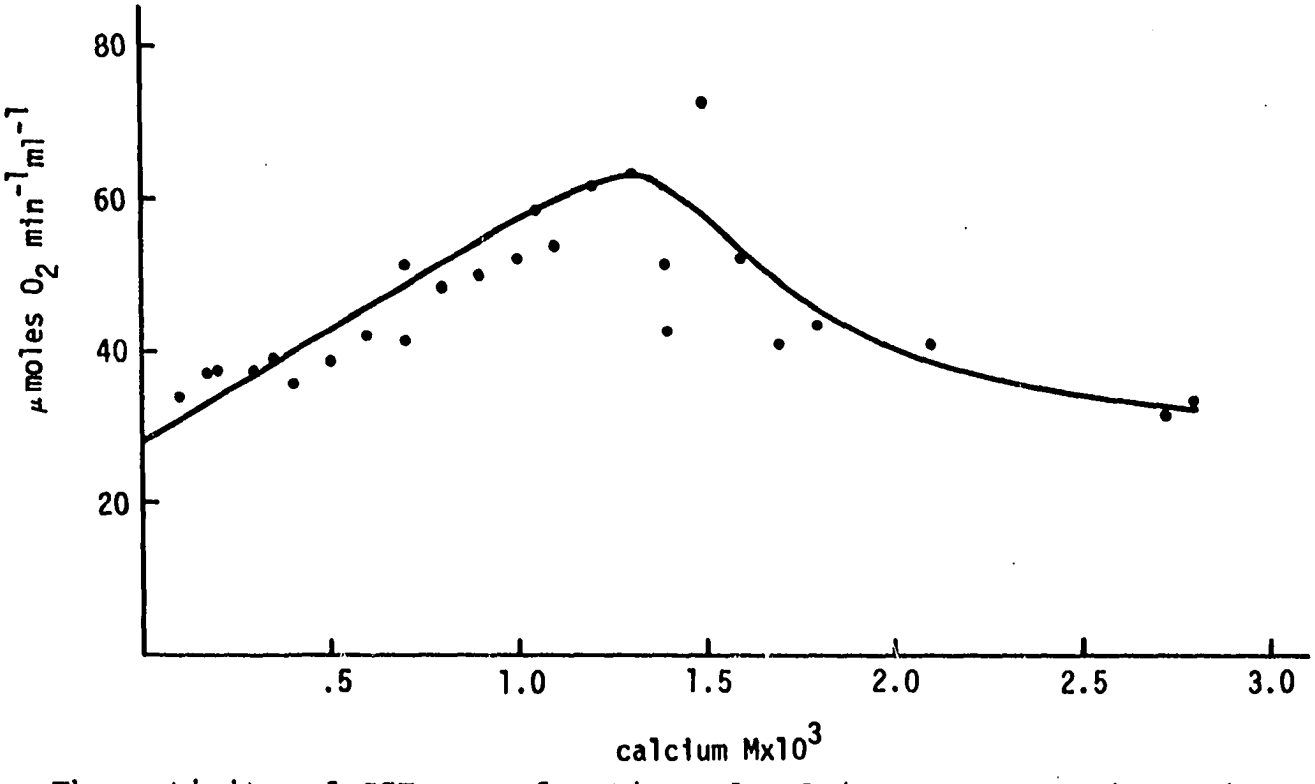


Figure 3. The activity of CSE as a function of calcium concentration, with a constant linoleic acid concentration of 7 x 10^{-4} M. The reaction was buffered in .2 M phosphate, pH 6.8

(1970) and confirmed here, the calcium when added after the initiation of the reaction of enzyme with substrate had no effect on the reaction rate. If calcium comes in contact with CSE before or concurrently with substrate, it increases the reaction rate.

Esterified substrates

Since Holman et al. (1969) postulated that a free carboxyl group on the substrate was necessary for calcium activation, methyl linoleate was used as substrate in some experiments. As seen in Table 2, the initial rate in the presence of calcium was about the same as in the absence of calcium. The same thing was found using trilinolein as substrate (Table 3). It is thus possible, as suggested by Holman, that the activation effect of calcium is partly the result of a facilitated substrate binding. The nonionic detergent Tween 20 activates the enzyme, presumably by solubilizing triglyceride, but calcium has no effect in the presence or absence of Tween 20. With linoleic acid, the activating effect of calcium is observed in the presence or absence of Tween 20.

Endogenous calcium in enzyme

Due to the fact that some lipoxygenase preparations are activated by calcium and some are not, I wanted to determine how much calcium was present endogenously in different enzyme

Table 2. The activity of CSE and lipoxygenase 2 with methyl linoleate as substrate, in the presence and absence of exogenous calcium. The reaction was buffered in .2 M Tris pH 8.0 or .2 M phosphate pH 6.8

CSE: Buffer	Calcium concentration	Methyl linoleate concentration	μmoles min^{-1} ml^{-1}
Tris	0	7×10^{-4} M	140
	7×10^{-4} M	7×10^{-4} M	125
	0	1.4×10^{-3} M	129
	1.4×10^{-3} M	1.4×10^{-3} M	119
Phosphate	0	7×10^{-4} M	92
	7×10^{-4} M	7×10^{-4} M	85
Lipoxygenase 2:			
Phosphate	0	7×10^{-4} M	.34
	7×10^{-4} M	7×10^{-4} M	.35

Table 3. The effect of calcium on the initial rate obtained with CSE, using trilinolein as substrate. The reaction was buffered in .01 M phosphate pH 8.0. Trilinolein concentration was 4.7×10^{-4} M and Tween 20, where present, was at .008%

Calcium concentration	Tween 20	μmoles min^{-1} ml^{-1}
0	-	13.4
1.4×10^{-3} M	-	14.8
0	+	29.0
0	+	27.0
1.4×10^{-3} M	+	28.0
1.4×10^{-3} M	+	28.0

preparations. The calcium contents and activities of several CSE preparations are given in Table 4. The 15 µg/ml calcium in .4 µl CSE would give a final calcium concentration in the reaction of 5×10^{-8} M. This is about four orders of magnitude less than the amount of exogenous calcium necessary to cause an activation. Dialysis of the CSE against water removed part of the calcium but not the activity. However, dialysis against EDTA removed all of the calcium and all of the activity. The activity of CSE could not be restored by removing EDTA and adding activating amounts of calcium. Calcium could not be removed by passage of the enzyme through

Table 4. The effect of dialysis on calcium content and activity of CSE. The reaction was buffered in .01 M phosphate pH 8.0

µl CSE	Treatment	µg Ca/ml	µmoles diene min^{-1} ml^{-1}
.04	undialyzed	47	32.5
.4	undialyzed	47	24.5
.4	dialyzed against H_2O	15	22.0
.04	dialyzed against EDTA, 2.5×10^{-2} M	0.0	0.0
.4	dialyzed against EDTA, 2.5×10^{-2} M	0.0	0.0
8.0	dialyzed against EDTA, 2.5×10^{-2} M	0.0	0.0

a Sephadex G-25 column, in contrast to the results of Koch et al. (1971) EDTA added to the reaction in a 66-fold molar excess over the endogenous calcium had no effect on the rate, as seen in Table 5. EGTA is similarly ineffective in reducing the activity in the absence of exogenous calcium. However, in the presence of exogenous calcium, EDTA had the effect which might be expected. The effect of calcium was reversed by the addition of EDTA equimolar with calcium.

Table 5. The effect of calcium and EDTA on CSE. The linoleic acid concentration was 7×10^{-4} M in a reaction buffer of .2 M Tris pH 8.0

Calcium	EDTA	μmoles O_2 min^{-1} ml^{-1}
Experiment 1:		
0	0	31.0
0	7×10^{-4} M	32.4
7×10^{-4} M	0	66.2
7×10^{-4} M	7×10^{-4} M	32.4
1.4×10^{-3} M	7×10^{-4} M	70.6
Experiment 2:		
0	0	33.2
1.4×10^{-3} M	0	51.9
1.4×10^{-3} M	1.4×10^{-3} M	29.5
7×10^{-4} M	0	64.8
7×10^{-4} M	7×10^{-4} M	33.2

Isoenzymes

Substrate specificity

Koch et al. (1958) reported the probable existence of two isoenzymes of soybean lipoxygenase, one more active on trilinolein than on linoleic acid, and the other having the reverse specificity. Christopher et al. (1970) isolated two isoenzymes, one having greatest activity on linoleic acid, the other having greater activity on either trilinolein or methyl linoleate than on linoleic acid. Since Restrepo (1971) had found calcium activation of lipoxygenase, but only when using CSE, it seemed reasonable that soybean contained a calcium-activated isoenzyme having some substrate specificity.

An experiment was done to compare the activities of CSE and a partially purified enzyme (40-80% ammonium sulfate fraction) on linoleic acid and on trilinolein. The results (Table 6) indicated that there are differences in the substrate specificity of the two preparations. The crude preparation contains a higher percentage of triglyceride activity than does the partially-purified enzyme. The CSE has about equal activity on both substrates but the partially purified enzyme has much more activity on the free fatty acid than on the triglyceride. I found that the CSE, as reported by Restrepo (1971), is activated by calcium at all

Table 6. The activity of CSE and a partially purified enzyme on linoleic acid and trilinolein. The reaction was buffered in .01 M phosphate pH 8.0

	Substrate and Concentration	μmoles O_2 min^{-1} ml^{-1}
CSE	linoleic acid 1.4 x 10^{-3}M	.94
	trilinolein 4.67 x 10^{-4} M	1.03
Partially-purified Enzyme	linoleic acid 1.4 x 10^{-3}M	3.4
	trilinolein 4.67 x 10^{-4}M	1.9

enzyme levels used (Figure 4). However, the partially-purified enzyme preparation exhibits the behavior shown in Figure 5. In this case, the response to calcium varies as a function of enzyme concentration. The enzyme is slightly activated by calcium at low enzyme concentration but is in-inhibited by calcium at high enzyme concentration. This observation may explain some of the conflicting claims for calcium activation or lack thereof. The same behavior seen in Figure 5 is also observed for other preparations produced by ammonium sulfate fractionation.

Lipoxygenases 1 and 2

In view of the foregoing and the reports by Yamamoto et al. (1970) and Christopher et al. (1970) on the presence of two isolable isoenzymes in soybean extracts, I decided to

Figure 4. The effect of calcium upon the initial rates obtained with various amounts of CSE. The reactions were run in .2 M Tris pH 8.0. Calcium, where present was equimolar with substrate, 7×10^{-4} M

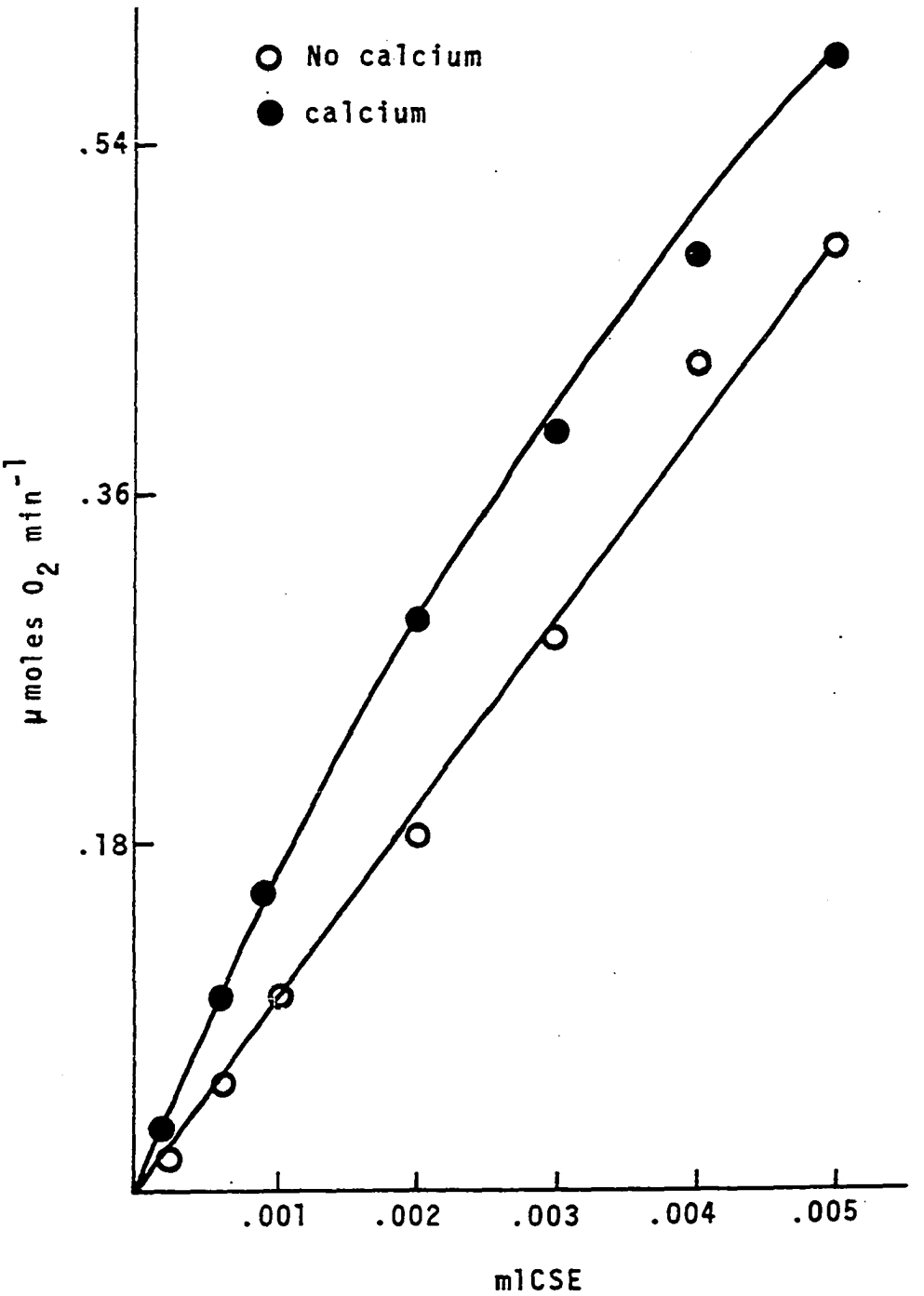
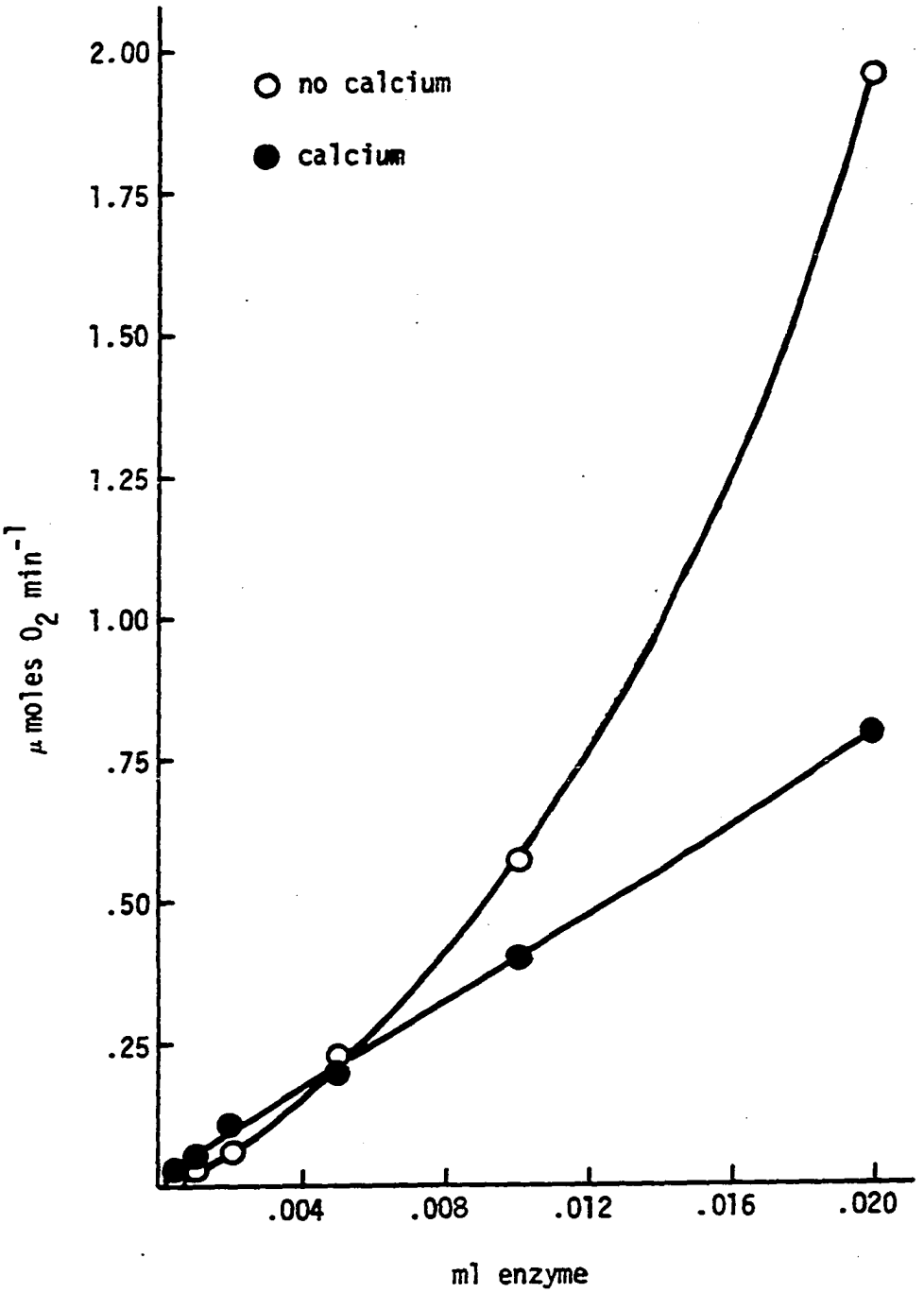

No calcium
calcium
.54
.36
.18
μmoles O_2 min^{-1}
.001
.002
.003
.004
.005
mlCSE

Figure 5. The effect of calcium on a partially-purified enzyme at different enzyme concentrations. The buffer was .01 M Tris pH 8.0 and the linoleic acid concentration was 1.4×10^{-3} M. Calcium, where added, was equimolar with substrate


2.00
1.75
1.50
1.25
1.00
.75
.50
.25
μmoles O_2 min^{-1}
no calcium
calcium
.004
.008
.012
.016
.020
ml enzyme

work with two isoenzymes isolated in our laboratory. The specific activity of the small peak from the DEAE-Sephadex column was several times less than that of the large peak, which came off the column later. The lipoxygenase 2 of the small peak rapidly lost activity with storage in the refrigerator. However, the activity of lipoxygenase 2 was increased with the addition of 1.4×10^{-3} M or 7×10^{-4} M calcium to the reaction mixture containing 7×10^{-4} M linoleic acid. As seen in Figure 6, calcium increased the initial rate at all enzyme levels used. The lipoxygenase 1 was slightly inhibited by calcium (Figure 7) under the same conditions giving activation with lipoxygenase 2. I have therefore confirmed the fact that two isoenzymes of soybean lipoxygenase have opposite responses to calcium. Yamamoto et al. (1970) showed calcium activation with lipoxygenase b at an activity level about two orders of magnitude lower than the activity levels where they showed calcium inhibition of lipoxygenase a. This, along with the finding shown in Figure 5 that both calcium activation and inhibition can be shown in the same preparation, opened the possibility that the different responses to calcium of the isoenzymes of Yamamoto et al. (1970) were due only to the amount of enzyme used. The results in Figures 6 and 7 refute this possibility.

Figure 6. The effect of calcium, equimolar with linoleic acid substrate, on lipoxygenase 2. Linoleic acid was present at a concentration of 7×10^{-4} M in .2 M Tris pH 8.0

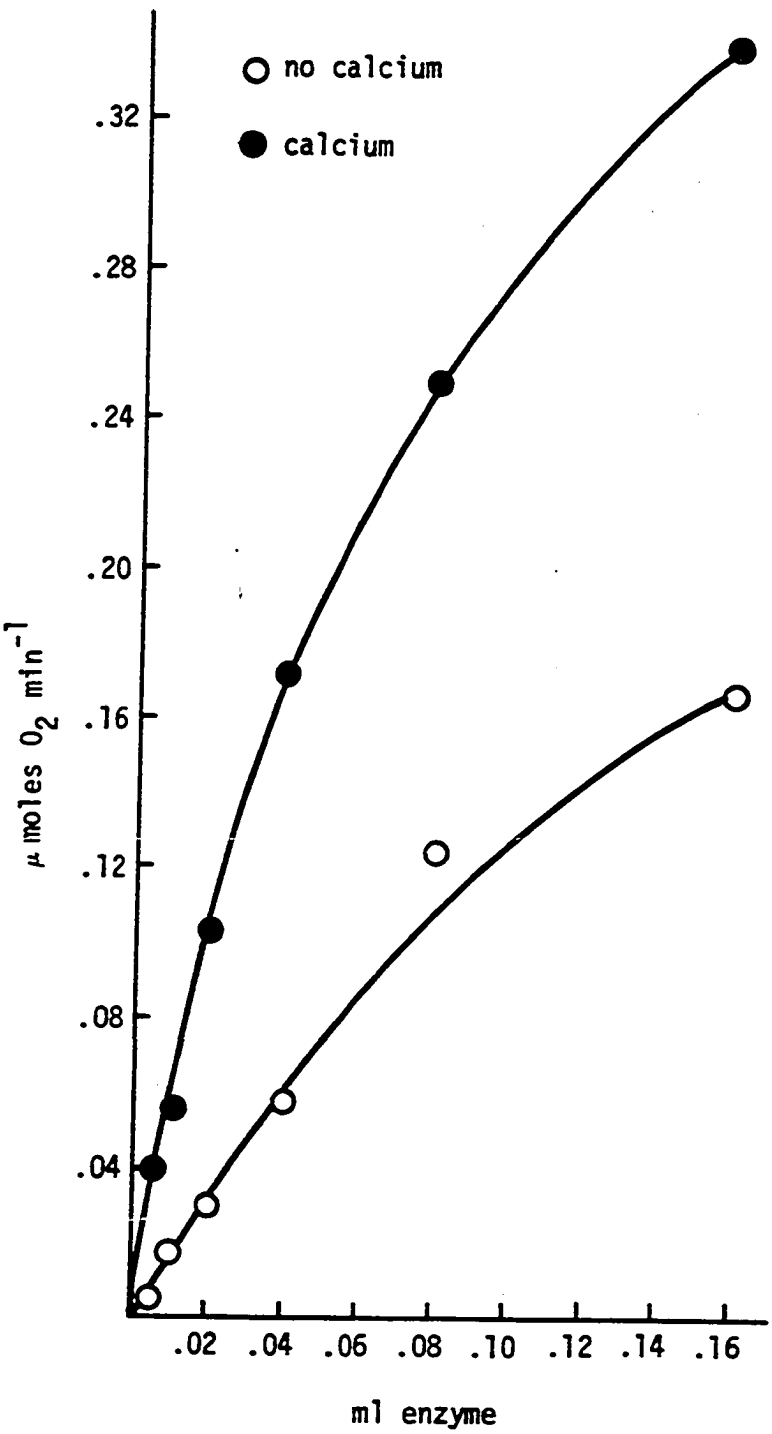
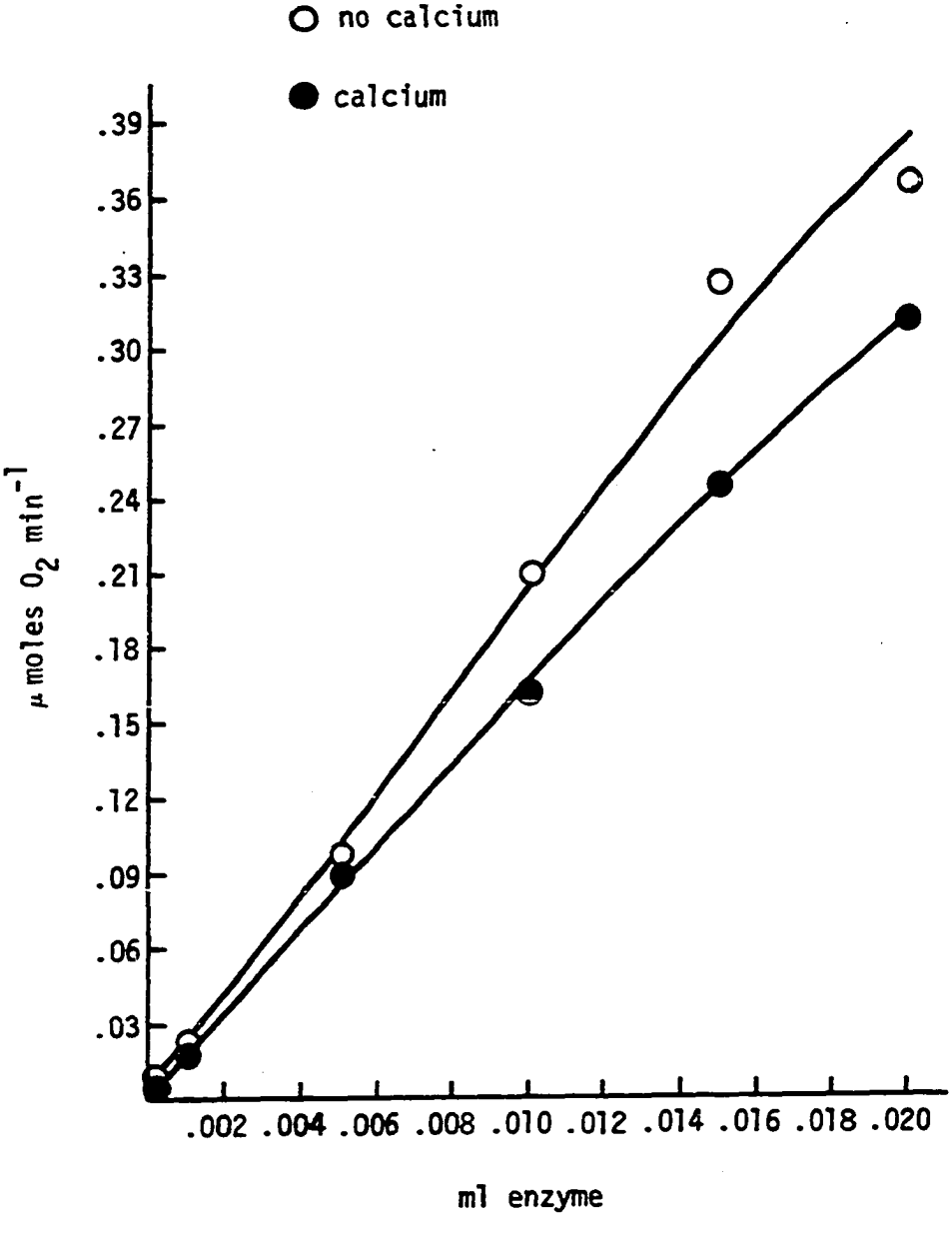

no calcium
calcium
.32
.28
.24
.20
.16
.12
.08
.04
μ moles O_2 min^{-1}
.02
.04
.06
.08
.10
.12
.14
.16
ml enzyme

Figure 7. The effect of calcium, equimolar with linoleic acid substrate, on lipoxygenase 1. Linoleic acid was present at a concentration of 7×10^{-4} M in .2 M Tris pH 8.0


no calcium
calcium
.39
.36
.33
.30
.27
.24
.21
.18
.15
.12
.09
.06
.03
μ moles O_2 min^{-1}
.002 .004 .006 .008 .010 .012 .014 .016 .018 .020
ml enzyme

Enzyme Destruction

Self-catalyzed enzyme destruction

I found that enzyme destruction was an important factor in many of my experiments. There appear to be two types of enzyme destruction. One type is the self-catalyzed destruction treated by Smith and Lands (1970, 1972). This type of enzyme destruction is readily seen over the course of an experiment (Figure 8 and Smith and Lands, 1970) particularly when using lipoxygenase 2 or very small amounts of other enzyme preparations. The reaction is reinitiated upon the addition of CSE showing that it is enzyme and not substrate that becomes limiting. I found that lipoxygenase 2 also exhibited this behavior and that the destruction was very close to first order (Figure 9). This is in agreement with Smith and Lands (1972). When this was discovered, I thought that calcium might be acting by hindering enzyme destruction. However, with calcium, the enzyme destruction rate is actually increased (Figure 9). This increased enzyme destruction rate is also seen for the partially-purified enzyme by measuring half-life of the enzyme as in Table 7. The half-life of the enzyme was the time it took for the velocity of the reaction to reach 50% of the initial velocity. This half-life was decreased with exogenous calcium present. If the kinetic mechanism for enzyme destruction of Smith and

Figure 8. Illustration of the self-catalyzed destruction of CSE during the reaction with substrate. After the velocity goes to zero, the reaction can be started again by the addition of a second enzyme aliquot, showing that the enzyme and not another component, has become limiting. The reaction was buffered in .01 M phosphate pH 8.0

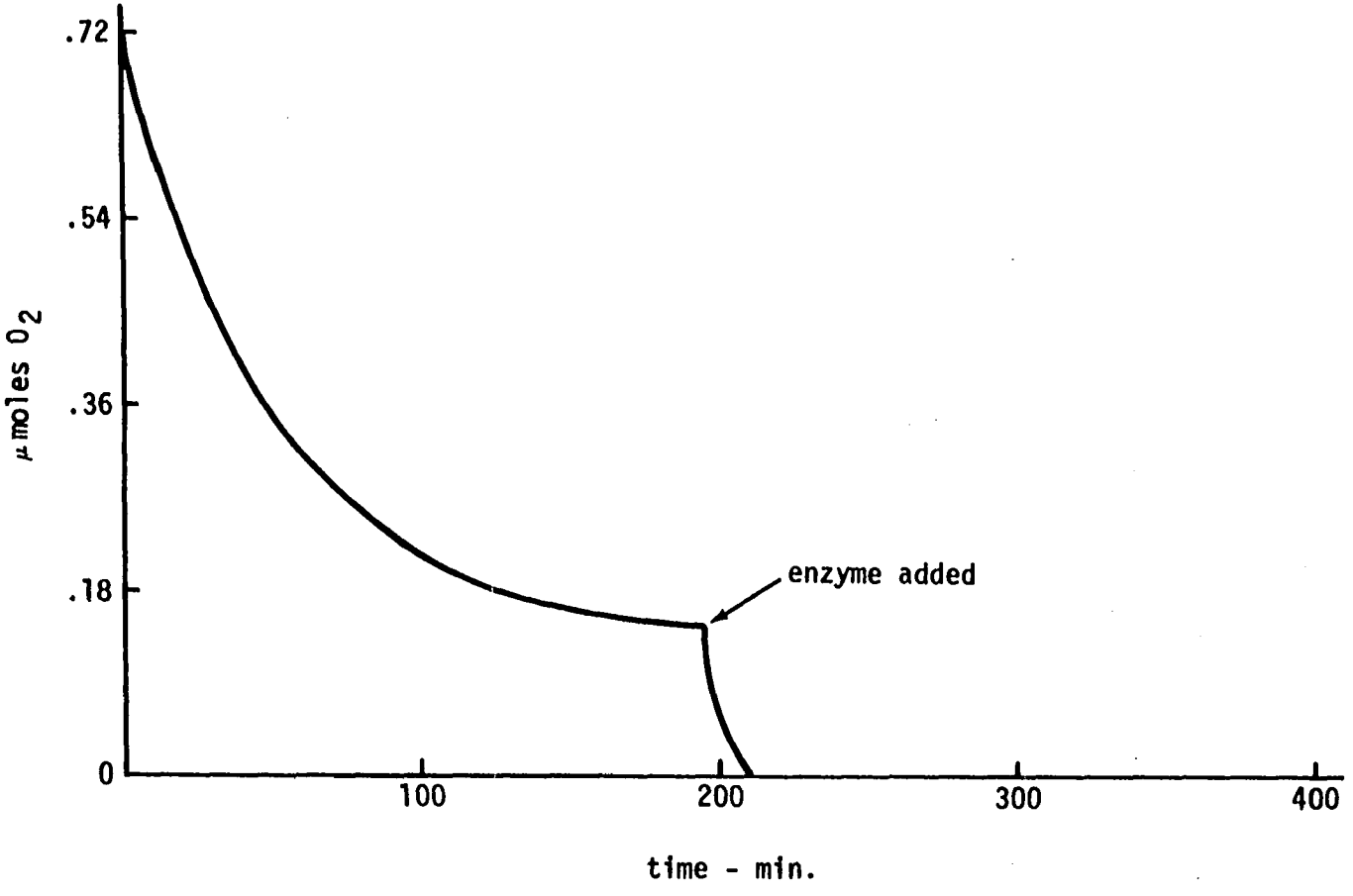

.72
.54
.36
.18
0
μmoles O_2
100
200
300
400
time - min.
enzyme added

Figure 9. The self-catalyzed destruction of lipoxygenase 2 during the reaction with linoleic acid present at a concentration of 1.4×10^{-3} M. Calcium, where present, was equimolar with substrate. The order of addition of components to the reaction is as indicated. The reaction was buffered in .2 M Tris pH 8.0. v_1 is the initial velocity and v_2 is the velocity at the indicated time

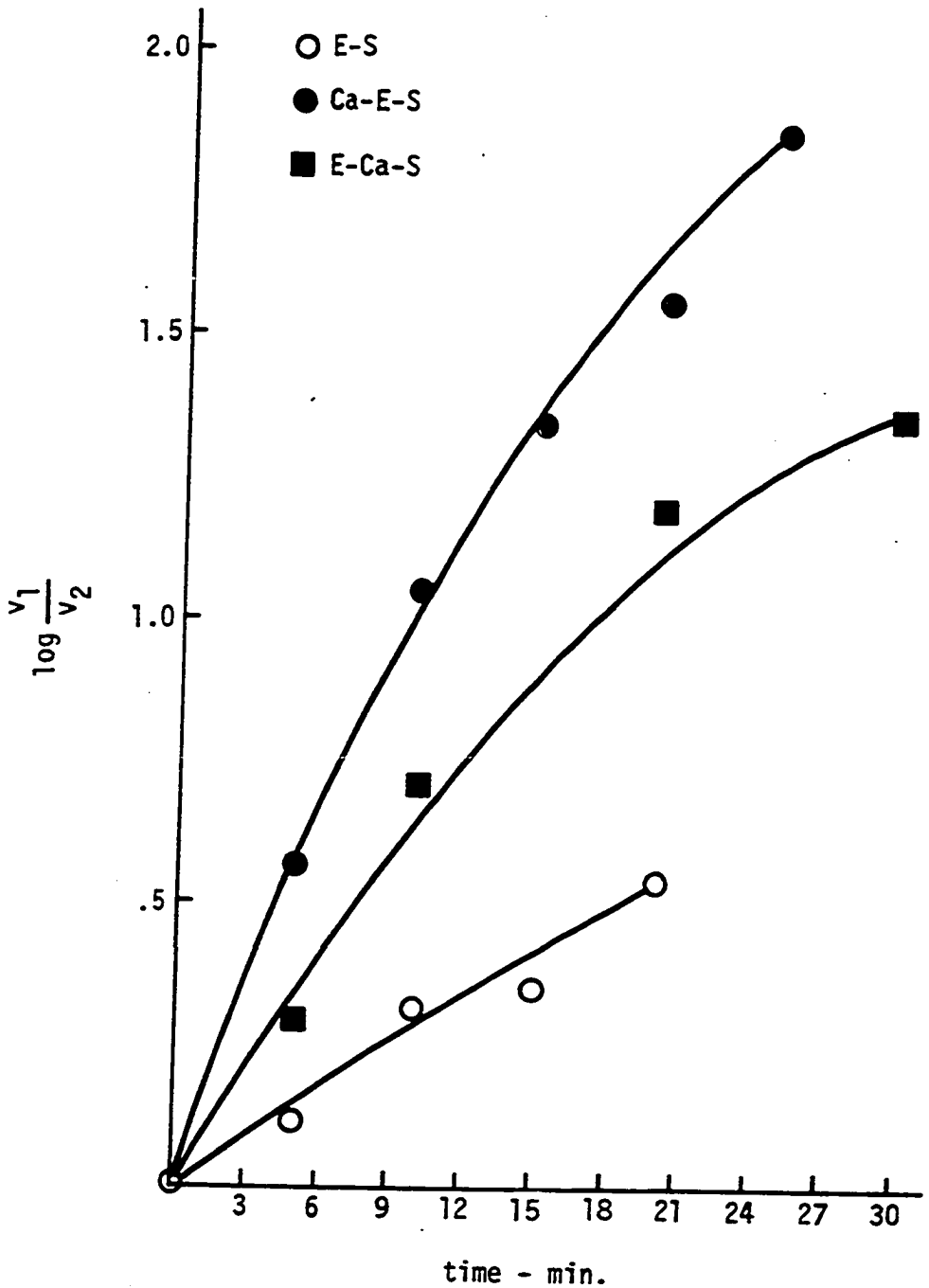

2.0
1.5
1.0
.5
E-S
Ca-E-S
E-Ca-S
log V1/V2
3
6
9
12
15
18
21
24
27
30
time - min.

Table 7. The effect of calcium on the self-catalyzed destruction of a partially purified enzyme catalyzing the linoleic acid reaction. The buffer was .01 M Tris pH 8.0

Ca^{++}	Linoleic	μmoles O_2 min^{-1} ml^{-1}	$t\frac{1}{2}$
0	1.4×10^{-3} M	1.10	32 min
1.4×10^{-3} M	1.4×10^{-3} M	2.75	12 min

Lands (1972) is correct, and enzyme must bind product before inactivation, the presence of calcium could increase the rate of enzyme destruction by increasing the rate of product formation.

Enzyme destruction in the absence of lipid substrate

Figure 10 shows that there is another source of enzyme destruction which has not been discussed in the literature. The curve in Figure 10 shows that the initial rate (19.7 μmoles O_2 min^{-1} ml^{-1}) obtained with CSE after incubating the CSE in the reaction mixture is lower than the initial rate (33.6 μmoles O_2 min^{-1} ml^{-1}) obtained without prior incubation (Figure 8). The reaction rate in the presence of substrate (and formed product), goes from 33.6 μmoles O_2 min^{-1} ml^{-1} to zero (Figure 8) in the time it takes for the enzyme in the absence of substrate and product to go to 19.7 μmoles O_2 min^{-1} ml^{-1} (Figure 10). This shows that not all the enzyme

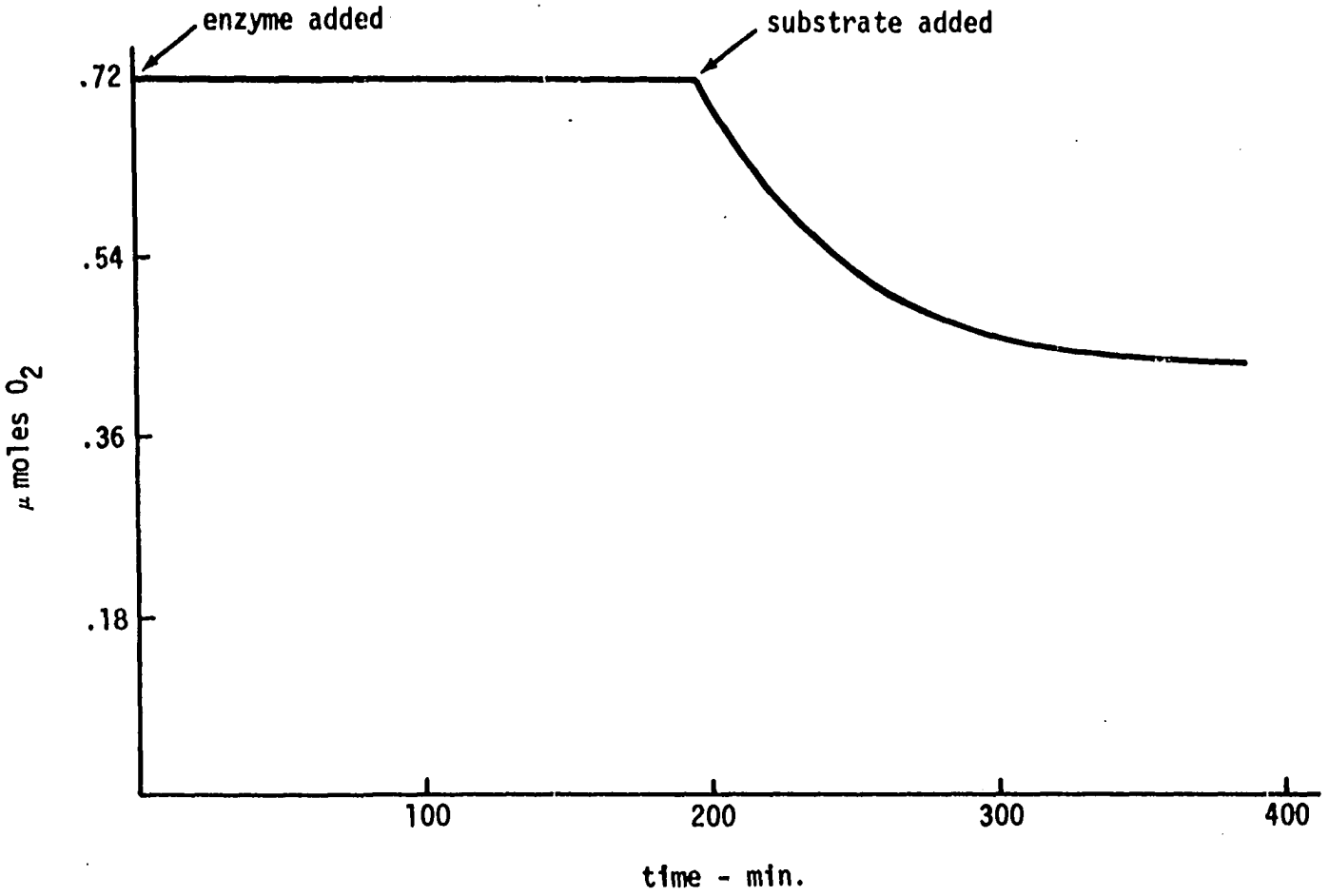


Figure 10. Illustration of the destruction of enzymatic activity of CSE in the absence of substrate and product. The initial rate is about half that shown in Figure 8, where there was no pre-incubation. The reaction was buffered in .01 M phosphate, pH 8.0

inactivation is due to the phenomenon treated by Smith and Lands (1972) but that some is due to another mechanism.

The destruction of the enzyme in the absence of substrate is shown by incubating the enzyme in the reaction buffer for various periods of time. As seen in Table 8, the loss of activity of partially-purified enzyme is not instantaneous upon dilution into the 3 ml reaction buffer but increases over time. Lipoxygenase 2 is not destroyed by dilution into the reaction buffer mixture.

Table 8. The effect on initial rate of pre-incubating the partially-purified enzyme for various periods of time in reaction buffer before the addition of substrate. The reaction buffer was .2 M Tris, pH 8.0. The substrate, linoleic acid, was present at a concentration of 1.4×10^{-3} M

Time of pre-incubation	μmoles O_2 min^{-1} ml^{-1}
0	27.8
1 minute	28.9
5 minutes	18.7
10 minutes	10.7

Cold-Insoluble Material

The addition of a second aliquot of partially-purified enzyme to a reaction mixture after the velocity has gone to zero results in a new initial velocity equal to the original initial velocity. Third and fourth enzyme aliquots result in the same initial velocity, indicating that this preparation did not contain a stable activator. Using CSE, the second enzyme aliquot gave a greater initial rate than the first, indicating that a stable activator is present in CSE. The CSE contains calcium, although in a smaller amount than is normally required to be added in order to get activation. Koch et al. (1971) claim that a cold-insoluble material is an activator for the reaction and contains calcium. I found that the cold-insoluble material of CSE contains calcium but that it also has most of the lipoxygenase activity. When this CSE was dialyzed against EDTA to remove calcium, no precipitate remained and there was no activity (Table 4). Dialysis against water, which removed part of the calcium, also removed part of the precipitate but did not have much effect on activity. This enzyme preparation then no longer responded to calcium. I postulate that lipoxygenase 2 gives CSE its response to calcium but is easily destroyed by dialysis so that CSE no

longer is activated by calcium. A report has appeared (Pistorius and Axelrod, 1973) that iron is present in lipoxygenase and that it is necessary for enzymatic activity. If this report is true, the effect of EDTA during prolonged dialysis may be due to the removal of iron.

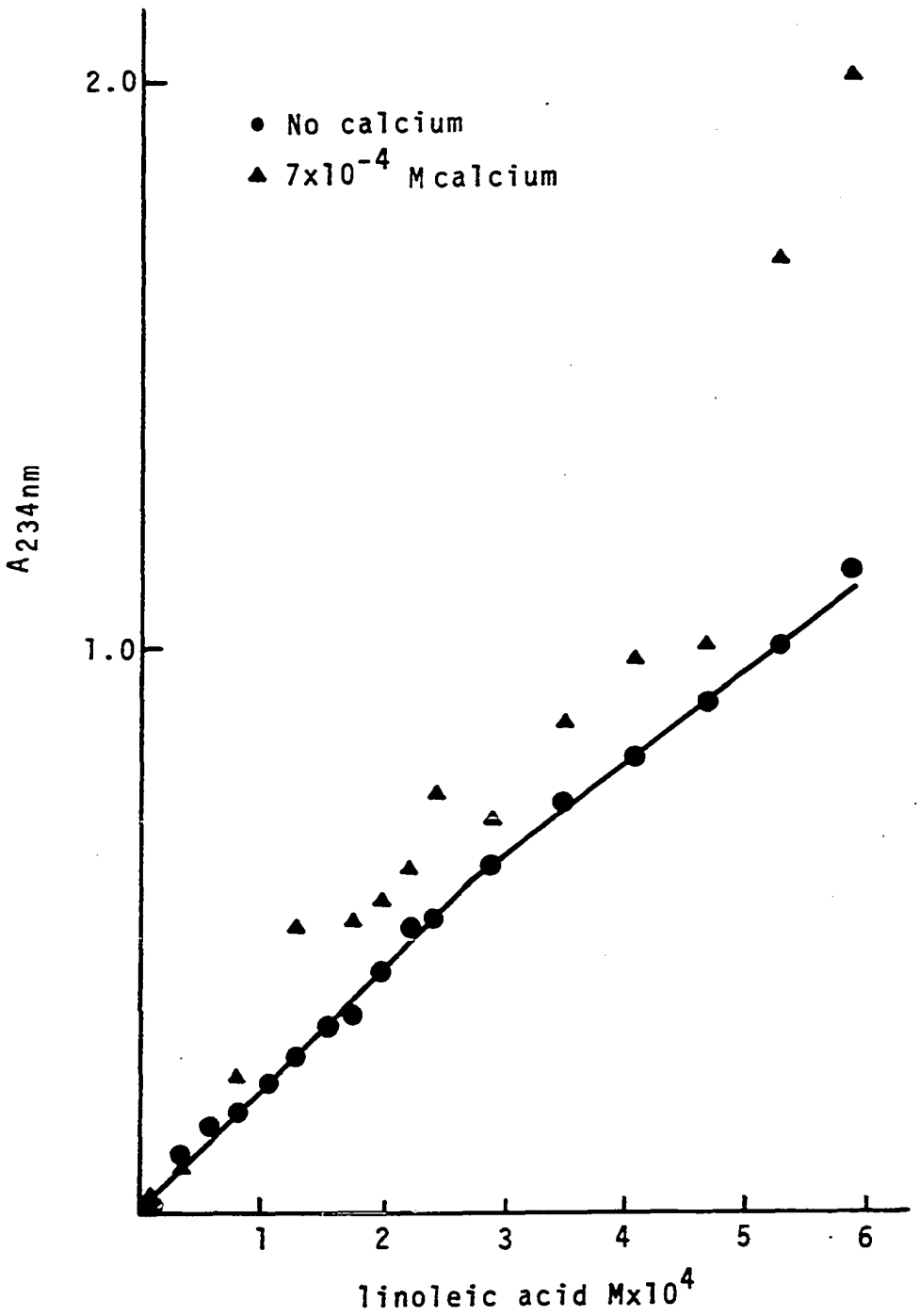
Physical State of Fatty Acid Substrate

Soaps of divalent cations are known to be insoluble and to form micelles more readily than soaps of monovalent cations (Kavanau, 1965). I made some efforts to determine the effect of calcium on the physical state of linoleic acid in the aqueous reaction buffers. A first approach to determining the physical state of the substrate is to determine its critical micelle concentration (CMC). This can be done, among other means, by plotting the absorbance of a surface-active chromophore as a function of concentration and determining the CMC as the concentration where the slope of the line changes (Duff and Giles, 1972). For the chromophore, I used the linoleic acid hydroperoxide present in small quantity due to autoxidation of linoleic acid. The phenomenon of change in slope was observed (Figure 11), so the hydroperoxide was apparently incorporated into micelles. The critical micelle concentration determined by this method was 2.7×10^{-4} M. The addition of calcium in

an enzyme-activating concentration (7×10^{-4} M) caused a change in the plot above a linoleic acid concentration of 6×10^{-5} M, due to some calcium-substrate interaction which caused a visible cloudiness and higher absorbancy readings than without calcium, especially above 5×10^{-4} M linoleate (Figure 11). Thus no CMC was determined by this method in the presence of calcium. The shape of the calcium curve in Figure 11 indicates that the amount of substrate as well as the calcium: substrate ratio is important in determining the physical state of the substrate in the presence of calcium. A high calcium: substrate ratio results in a large amount of light scattering if the substrate concentration is high enough. It also results in a lower initial rate (Figure 3).

Ultracentrifugation at 260,000 g of 1.4×10^{-3} M linoleate containing hydroperoxide chromophore showed that the micelles were less dense than the aqueous buffer, .2 M Tris pH 8.0. However, in the presence of 1.4×10^{-3} M $CaCl_2$, the chromophore sedimented. This was apparently a two-phase sedimentation, the light-scattering material centrifuging out within 8 minutes after attainment of speed and then some other phase sedimenting slowly over the course of the 6-hour run. It is thus obvious that calcium is causing

Figure 11. Absorbancy at 234 nanometers as a function of linoleic acid concentration of a solution of linoleic acid containing linoleic acid hyperperoxide as a contaminant. The buffer used was .2 M Tris HCl pH 8.0


2.0
1.0
● No calcium
▲ 7x10⁻⁴ M calcium
A234nm
1
2
3
4
5
6
linoleic acid Mx10⁴

a physical change in the substrate. The mechanism of the effect of these physical changes on the enzymatic rate has not been determined.

SUMMARY AND CONCLUSIONS

Evidence reported in this dissertation confirms the results of Yamamoto et al. (1970) that two soybean lipoxygenase isoenzymes have opposite responses to calcium added to the reaction mixture. In addition, the isoenzymes used in the present study correspond to those of Christopher et al. (1970). Why the two isoenzymes show opposite responses to calcium is as yet unanswered. The calcium activation of CSE is probably due simply to its content of lipoxygenase 2. This conclusion is supported by the data showing the removal of the calcium activation property of CSE by dialysis against water as well as the demonstrated lability of lipoxygenase 2.

Calcium does not act by protecting lipoxygenase from the "self-catalyzed" destruction. In fact, data show that the rate of destruction of lipoxygenases 1 and 2 is increased in the presence of calcium. However, in the presence of calcium, the rate of the enzyme-catalyzed reaction is greater throughout the course of the enzyme reaction and the reaction is completed sooner.

In this dissertation, I report for the first time the destruction of lipoxygenase 1 in the absence of substrate and product. The destruction occurs upon the dilution of lipoxygenase 1 into the reaction mixture and is progressive over

time. This type of destruction is not observed in the case of lipoxygenase 2. The activity of CSE is removed by dialysis against EDTA. The addition of calcium does not restore activity to CSE.

Several lines of evidence point to the interaction of calcium with substrate. I found that only when the substrate had a free carboxyl group was calcium effective in activating the enzyme. The effect of calcium on substrate is visible to the unaided eye and therefore involves a phenomenon different from an effect on CMC. The optimum calcium concentration resulting in maximum lipoxygenase activity varies with substrate concentration and does not vary with enzyme concentration. However, the 1:1 relationship between calcium and substrate suggested by Restrepo *et al.* (1973) does not hold. The physical state of the substrate changes with the addition of calcium, but the effect, if any of this change upon the enzymatic reaction is unknown. It is probable that some type of enzyme-substrate-calcium complex is formed, possibly with calcium as a bridge between enzyme and substrate. The substrate form for interaction with enzyme may be monomer or dimer (Goodman, 1958) in equilibrium with micelles or insoluble substrate so that the physical state of the substrate *per se* has little effect on the enzymatic reaction.

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